

Immunity

Supplemental Information

**The MicroRNA-132 and MicroRNA-212 Cluster
Regulates Hematopoietic Stem Cell Maintenance
and Survival with Age by Buffering FOXO3 Expression**

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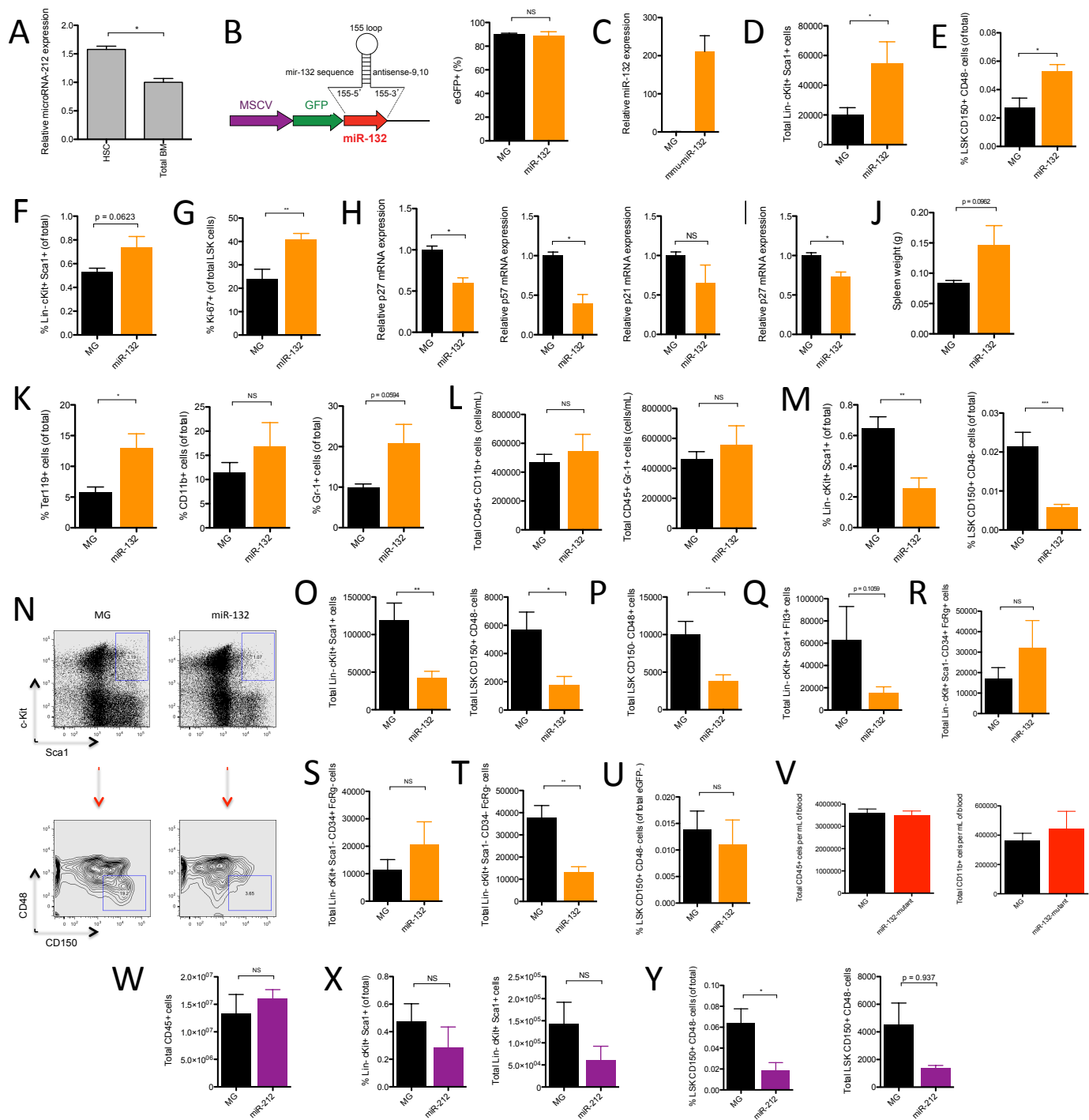


Figure S1, related to Figure 1: Ectopic expression of microRNA-132 leads to deregulated hematopoiesis. (A) miR-212 expression in total bone marrow and HSCs. (B) Schematic of the retroviral construct used to over-express miR-132 and reconstitution efficiency of mice transplanted with MG or miR-132 donor bone marrow. Peripheral blood was analyzed at 8-weeks post-reconstitution for the proportion of eGFP⁺ CD45⁺ leukocytes. (C) Expression of miR-132 in bone marrow cells obtained from MG and miR-132 mice. Data is represented as mean \pm SEM. (D) – (U) WT C57BL/6 mice were lethally irradiated and reconstituted with donor bone marrow cells expressing either a control (MG) or a miR-132 over-expressing (miR-132) retroviral vector (n=8-12 mice per group). Data represents at least three independent experiments and is shown for 16-weeks post-reconstitution unless otherwise specified. (D) Total number of LSK cells in the bone marrow at 8-weeks post-reconstitution. (E) Frequency of HSCs in the bone marrow at 8-weeks post-reconstitution. (F) Frequency of LSK cells in the bone marrow at 8-weeks post-reconstitution. (G) Percentage of Ki-67⁺ cells within the bone marrow LSK at 8-weeks post-reconstitution. (H) p27, p57, and p21 transcript expression in the bone marrow at 2-months post-reconstitution. (I) p27 transcript expression in the bone marrow compartment at 4-months post-reconstitution. (J) Spleen weights. (K) Percentage of Ter119⁺, CD11b⁺ and Gr-1⁺ cells in the spleen. (L) Total number of CD11b⁺ and Gr-1⁺ cells in the peripheral blood. (M) Representative FACS plot and gating for HSCs (Lineage⁻ Sca1⁺ cKit⁺ CD150⁺ CD48⁻). (N) Percentage of LSK cells and HSCs in the bone marrow. (O) Total number of LSK cells and HSCs at 9-months post-reconstitution. Total number of (P) multipotent progenitors (MPPs), (Q) lymphoid primed MPPs (LMPPs), (R) granulocyte-monocyte progenitors (GMPs), (S) common myeloid

progenitors (CMPs), and (T) myeloid-erythroid progenitors (MEPs) in the bone marrow. (U) Percentage of HSCs in the bone marrow within the eGFP- population. (V) WT C57BL/6 mice were lethally irradiated and reconstituted with donor bone marrow cells expressing either a control (MG) or a miR-132-mutant over-expressing (miR-132-mutant) retroviral vector in which the miR-132 seed sequence was mutated (n=5 mice per group). Graphs show total peripheral blood CD45+ leukocytes and CD11b+ cells at 9-months post-reconstitution. (W) – (Y) WT C57BL/6 mice were lethally irradiated and reconstituted with donor bone marrow cells expressing either a control (MG) or a miR-212 over-expressing (miR-212) retroviral vector (n=4 mice per group). (W) Total bone marrow CD45+ cells. (X) Frequency and total number of LSK cells in the bone marrow. (Y) Frequency and total number of HSCs in the bone marrow. Data is represented as mean \pm SEM. * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$ using a Student's *t* test.

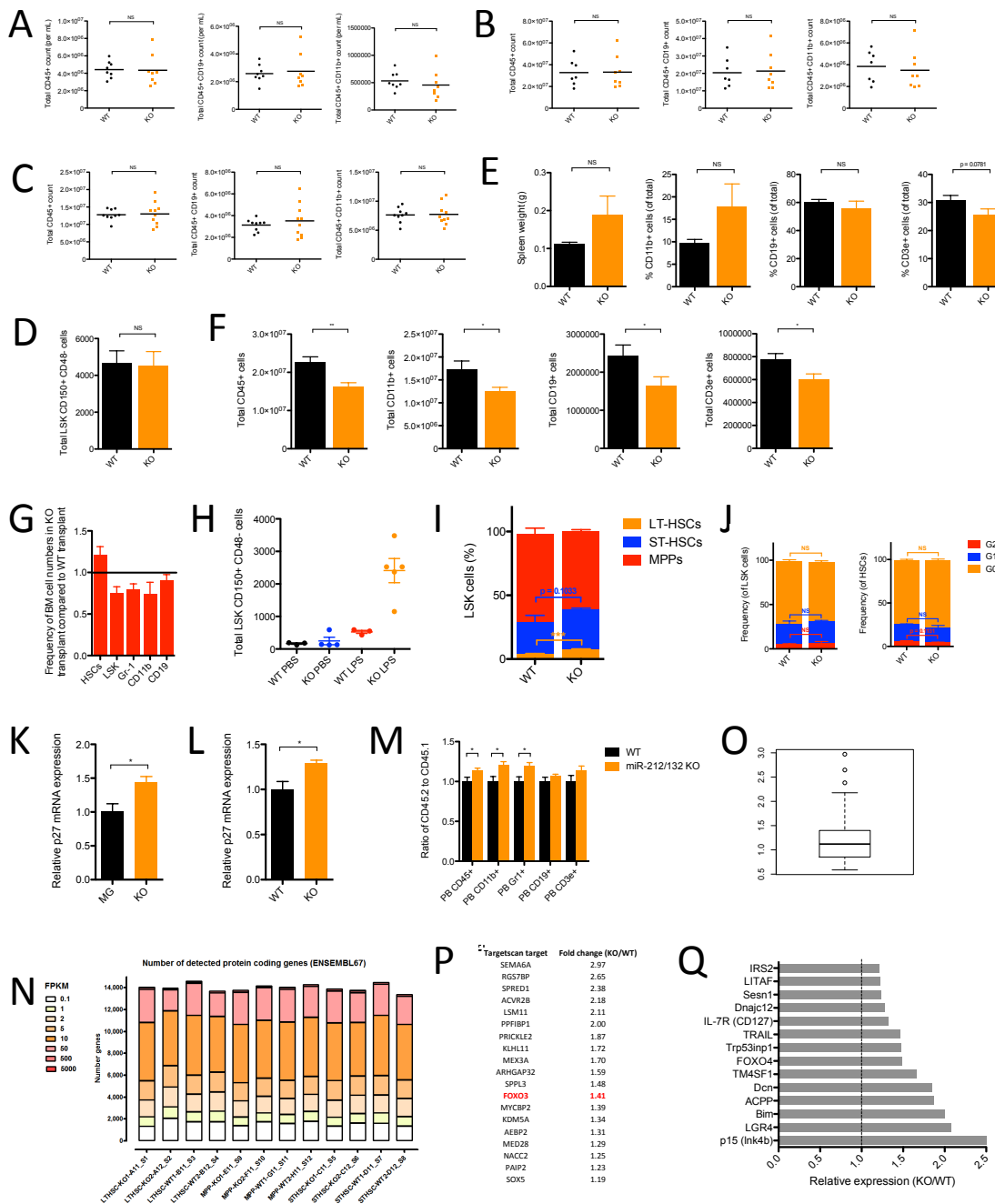


Figure S2, related to Figures 2 and 3: Genetic deletion of Mirc19 in mice leads to deregulated hematopoiesis with age. (A) – (C) Mice with a genetic deletion of Mirc19 (*Mirc19*^{-/-}) along with WT mice in the C57BL/6 background were analyzed to understand the physiological contribution of Mirc19 on hematopoietic output (n=7-8 mice per group). (A) Total number of peripheral blood CD45+, CD19+ and CD11b+ cells at 12-weeks of age. (B) Total number of splenic CD45+, CD19+ and CD11b+ cells at 12-weeks of age. (C) Total number of bone marrow CD45+, CD19+ and CD11b+ cells at 12-weeks of age. (D) Total number of HSCs in the bone marrow at 12-weeks of age. (E) Spleen weight, and percentage of splenic CD11b+, CD19+ and CD3e+ cells at 60-64 weeks of age. (F) Total number of CD45+, CD11b+, CD19+ and CD3e+ cells in the bone marrow at 60-64 weeks of age. (G) WT or *Mirc19*^{-/-} (KO) bone marrow cells were transplanted into lethally irradiated C57BL/6 mice. The relative ratio (KO/WT) of total numbers of various cell populations in the bone marrow of these mice at 60-weeks post-reconstitution is shown. (H) – (J) 6-month old WT and *Mirc19*^{-/-} mice were treated with 9 evenly-spaced low-dose (1mg/kg of body weight) LPS or PBS injections over one month. (H) Total number of HSCs in the spleen. (I) Relative proportions of early progenitors in the bone marrow. (J) Proportion of bone marrow LSK cells and HSCs in each stage of the cell cycle (G0, G1, G2/M). (K) Expression of p27 in the bone marrow compartment. (L) Expression of p27 in the bone marrow compartment of mice 5-days after a single treatment of 5-fluorouracil. (M) WT or *Mirc19*^{-/-} CD45.2+ bone marrow cells from primary transplant experiments, calibrated for the total number of phenotypically defined HSCs, were injected in a 1:1 ratio with CD45.1+ WT bone marrow cells into irradiated C57BL/6 CD45.2+ recipients. The graph represents the relative ratio of mature cells in

the peripheral blood of these secondary transplant mice at 16-weeks post-reconstitution.

(O) Summary of the number of detected protein coding genes and FPKM values for sequenced samples. LT-HSC: long-term HSCs, LSK CD150⁺ CD48⁻. SH-HSCs: short-term HSCs, LSK CD150⁻ CD48⁻. MPPs: multipotent progenitors, LSK CD150⁻ CD48⁺. WT: wildtype sample. KO: *Mirc19*^{-/-} sample. (O) Box-plot showing enrichment of miR-132 targets in miR-212/132^{-/-} HSCs compared to WT HSCs. (P) Fold-change of several miR-132 targets in miR-212/132^{-/-} HSCs compared to WT HSCs. (Q) Fold-change in several FOXO3 regulated genes in miR-212/132^{-/-} HSCs compared to WT HSCs. (R) Fold-change in several autophagy related genes in miR-212/132^{-/-} HSCs Data represents at least two independent experiments and is represented as mean \pm SEM. * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$ using a Student-T test.

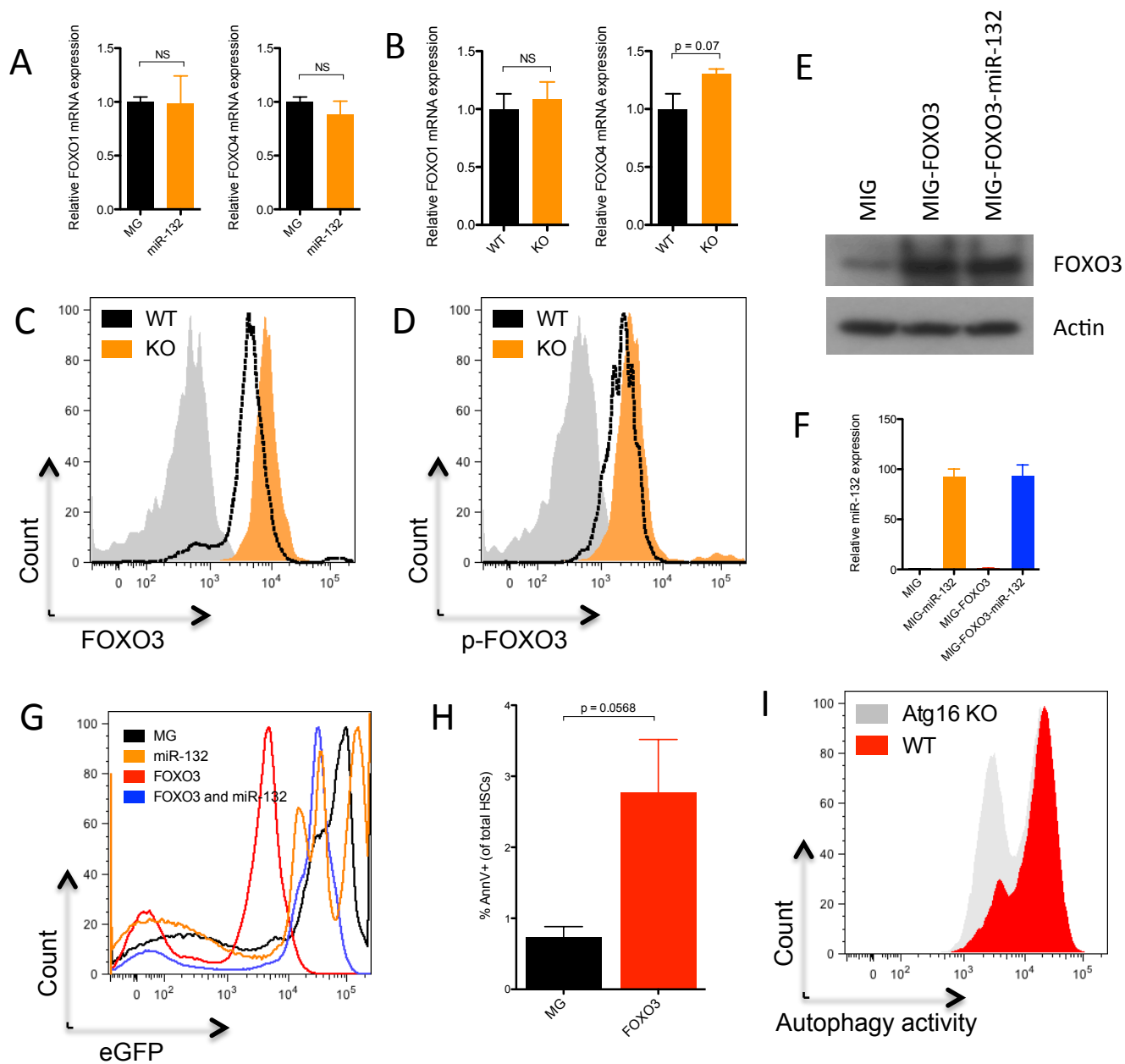


Figure S3, related to Figures 6 and 7: Validation of microRNA-132 and FOXO3 over-expression in FOXO3 rescue experiment. (A) FOXO1 and FOXO4 expression in total bone marrow cells from WT^{MIG} and WT^{miR-132} mice. (B) FOXO1 and FOXO4 expression in lineage-depleted bone marrow cells from WT and miR-212/132^{-/-} mice. RNA was obtained from the respective cell populations and FOXO1 and FOXO4 expression was quantified by RT-qPCR (n=2-3 biological replicates). (C) Intracellular staining analyzed by FACS for FOXO3 protein expression in bone marrow HSCs obtained from WT and miR-212/132^{-/-} mice and starved for 12 hours *in-vitro*. (D) Intracellular staining analyzed by FACS for phosphorylated FOXO3 (p-FOXO3) protein expression in bone marrow HSCs obtained from WT and miR-212/132^{-/-} mice and starved for 12 hours *in-vitro*. (E) – (F) Bone marrow expressing either a control (MIG), a miR-132 over-expressing (MIG-miR-132), a FOXO3 over-expressing (MIG-FOXO3), or a FOXO3 and miR-132 over-expressing (MIG-FOXO3-miR-132) retroviral vector were analyzed using Taqman RT-qPCR for miR-132 expression or Western Blot for FOXO3 protein expression. (E) FOXO3 protein expression in bone marrow cells transduced with the indicated retroviral vectors. (F) Mature miR-132 expression in in bone marrow cells transduced with the indicated retroviral vectors. (G) eGFP expression in the bone marrow compartment of mice reconstitution with bone marrow cells transduced with the indicated retroviral vector. (H) AnnexinV staining performed on bone marrow HSCs from MIG and MIG-FOXO3 mice (n=3). (I) Detection of autophagy activity using the Cyto-ID autophagy detection assay in bone-marrow-derived dendritic cells treated with Rapamycin and obtained from WT and Atg16 deficient (Atg16 KO) mice. Data represents at least two independent experiments and is represented as mean ± SEM.

Supplemental Experimental Procedures

DNA Constructs

The miR-132 over-expression construct was cloned into the MSCV-eGFP (MG) vector. In this modified vector, MG-miR-132, containing an MSCV promoter, the eGFP is placed immediately downstream of the 5' LTR and the miR-132 expression cassette is placed immediately downstream of the eGFP stop codon. A miR-132-mutant vector was similarly constructed with a mutated miR-132 seed sequence. For FOXO3 rescue experiments, FOXO3 cDNA was cloned into the MSCV-IRES-eGFP (MIG) vector, immediately downstream of the 5' LTR and upstream of the IRES. miR-132 was cloned downstream of eGFP as described above. For FOXO3 knockdown experiments, TagBFP was first subcloned into the MG vector, creating MSCV-TagBFP (MB). Several FOXO3 shRNA sequences were designed using the Invitrogen Block-iT RNAi Designer and cloned in the microRNA-155 loop-and-arms format immediately downstream of TagBFP in the MB vector.

Cell sorting for RNA extraction

Bone marrow cells were harvested from WT C57BL/6 mice and depleted of red blood cells (RBCs) using RBC lysis buffer (BioLegend). Cells were then spun down, resuspended in MACS separating buffer (Phosphate buffered saline, pH7.2, with 0.5% BSA and 2mM EDTA) and filtered through a 70uM cell-strainer. These cells were then blocked with FcBlock (Becton Dickinson) and depleted of mature cells on a magnetic column using biotin-conjugated mouse antibodies for CD3e, CD8, CD4, CD19, B220, CD11b, Gr-1, IL-7Ra, and Ter119 (BioLegend) and streptavidin magnetic beads

(Miltenyi), as suggested by the manufacturer (Miltenyi). Cells were subsequently stained with fluorophore-conjugated antibodies for lineage markers (CD3, CD19, CD11b, Gr-1, Ter119, Nk1.1), cKit, Sca1, CD150, CD48 and with 7-AAD, and several populations were sorted for analysis including HSCs (Lineage- cKit⁺ Sca1⁺ CD150⁺ CD48⁻), LSK cells (Lineage- cKit⁺ Sca1⁺), and other progenitor subsets as detailed in the text. Cells were sorted on a FACS Aria IIu cell sorter (Becton Dickinson) at the Caltech Flow Cytometry Core Facility. Mature cell populations were sorted using a magnetic column as described above using positive-selection for the respective surface marker (Gr-1, CD11b, CD19). All cells were lysed using Qiazol lysis buffer (Qiagen) and processed using the miRNAeasy RNA prep kit (Qiagen). RNA was then subjected to qPCR.

Sample preparation for RNA-sequencing

LT-HSCs (LSK CD150⁺ CD48⁻), ST-HSCs (LSK CD150⁻ CD48⁻) and MPPs (LSK CD150⁻ CD48⁺) from WT and miR-212/132^{-/-} were sorted as described above. Cells were sorted directly into cell lysis buffer and processed using an RNAeasy kit (Qiagen) with *DNaseI* digestion (Qiagen) as per manufacturer's protocol. Libraries were prepared using the SMART-seq2 protocol (Picelli et al., 2013) modified to use Maxima H Minus enzyme for reverse transcription (Thermo Scientific) (Satija et al., 2015). Amplified cDNA products were purified using AMPure XP SPRI beads (Beckman Coulter) and eluted in TE buffer (Teknova). Cleaned-up amplified cDNA was used for library construction using the Nextera XT DNA Sample Preparation Kit and Nextera XT Index Kit (Illumina). Libraries were then pooled and cleaned-up using AMPure XP SPRI beads

(Beckman Coulter Genomics). Final library quality and quantity were assessed using a DNA High Sensitivity chip (Agilent).

RNA-seq data generation and analysis

Libraries were sequenced on the Illumina HiSeq 2500. Paired-end 2x25bp reads were generated. The reads were filtered for rRNA contamination by aligning against mouse ribosomal sequences using Bowtie (version 0.12.7) (Langmead et al., 2009), and retaining unaligned read pairs. The refSeq annotation for the mm9 version of the mouse genome was used to create a transcriptome Bowtie index, to which read pairs were aligned with the following settings: “-v 2 -a -X 1000”. Gene expression levels were estimated using eXpress (version 1.5.1) (Roberts and Pachter, 2013), and the effective count values were used as input to DESeq (Anders and Huber, 2010) for evaluating differential expression. The targets of miR-132 were obtained from TargetScan (mouse release 6.2) (Friedman et al., 2009).

Immunoblotting

Pelleted cells were resuspended in RIPA lysis buffer (Sigma) containing protease inhibitors for 20 minutes on ice. Samples were spun down at max speed at 4°C for 10 minutes and the supernatant was processed immediately or flash-frozen on dry ice for analysis in the future. Total cell extracts were fractionated by gel-electrophoresis on a mini-PROTEAN TGX gradient (4-15%) gel (Bio-Rad) and electroblotted onto a PVDF membrane using a wet transfer apparatus (Bio-Rad). Protein detection was subsequently performed with the following antibodies: FoxO3a (75D8) (Cell Signaling Technologies),

p27-HRP (sc-538) (Santa Cruz Biotechnology), actin-HRP (sc-1616) (Santa Cruz Biotechnology) and goat anti-rabbit-IgG-HRP (sc-2030) (Santa Cruz Biotechnology).

Mice

All mice used in this study are of the C57BL/6 background. The miR-212/132^{-/-} mice were generated previously and are described elsewhere (Ucar et al., 2012). Briefly, these mice were generated by homologous recombination targeting the genomic cluster containing both microRNA-212 and microRNA-132. Mice obtained were backcrossed to the C57BL/6 background for at least 20 generations prior to the initiation of the reported studies.

Bone marrow reconstitution

WT C57BL/6 or miR-212/132^{-/-} mice were treated with 5-fluorouracil (10ug; Sigma) for 5 days to enrich for hematopoietic stem and progenitor cells (HSPCs) in the bone marrow. After 5 days, bone marrow cells were harvested, red blood cells (RBCs) were lysed with RBC lysis buffer (BioLegend), and cells were plated in HSPC media which was comprised of complete RPMI with mouse SCF (50 ng/mL), IL-3 (20 ng/mL), and IL-6 (50 ng/mL). Cells were then cultured in 24-wel plates for 24 hours and spin-infected with PCL-ecotropic pseudotyped gamma-retrovirus expressing the construct of interest, which was either a microRNA, shRNA or a gene, as described in under the *DNA Constructs and Primers* section of the main text. Spin-infections were performed by removing supernatant carefully from cell culture plates and adding virus with 8 ug/mL Polybrene (Santa Cruz Biotechnology). Plates

were then placed in a centrifuge for 2 hours at 30°C and 2500RPM. Immediately following infection, virus supernatant was removed and replaced with HSPC media. 24 hours later a second identical spin infection was performed. After another 24 hours, recipient mice were lethally irradiated (1000 rads from Cs137 source) and 250,000 to a million virus-infected HSPCs were retro-orbitally delivered to reconstitute the immune system. Recipients were maintained on Septra and in autoclaved cages for at least one month post-reconstitution.

Virus production

To generate retrovirus for HSPC infection, 10 million HEK293T cells were first plated in a 15cm plate. 24 hours later, cells were transfected with both the pCL-Eco vector and either the pMG vector or the relevant variant described above for gene delivery. For transfection, we used BioT (Bioland Scientific) as per the manufacturers protocol. 36 hours after transfection, virus was collected, filtered through a 45uM syringe filter, and used for infection of HSPCs.

Flow cytometry

Cells were stained with fluorophore-conjugated antibodies (all from BioLegend unless indicated) for CD45.1, CD45.2, CD11b, Gr-1, CD19, B220, CD3e, Nk1.1, Ter119, cKit, Sca1, CD150, CD48, EPCR (Ebioscience), CD34, Flt3, FcRg or IL-7Ra in various combinations to characterize relevant hematopoietic cell populations. Intracellular staining was performed by first performing surface staining of cells, followed by fixation and permeabilization (Cytofix/Cytoperm kit; BD Biosciences) and subsequent staining

with either Ki67 (BioLegend) and Hoescht33342 (Life Technologies) for cell-cycling analysis, or an anti-FoxO3a (75D8) (Cell Signaling Technologies) or anti-FoxO3a (phosphor S253) (Abcam) primary antibody followed by an anti-Rabbit-IgG secondary antibody conjugated to Alexafluor488 (Cell Signaling Technologies). Samples were analyzed on a MACSQuant10 Flow Cytometry machine (Miltenyi). Gating and analysis was performed using FlowJo software.

Autophagy and reactive-oxygen species assays

HSCs were sorted as described above from either WT or miR-212/132^{-/-} C57BL/6 mice, or from reconstituted mice with donor WT or miR-212/132^{-/-} bone marrow infected with either MB or shFOXO3 retroviral constructs. For all in-vitro experiments, cells were sorted directly in a 96-well plate containing cell culture media with the appropriate growth factors and cytokines (all from Ebioscience). For caspase activation assays and reactive oxygen species detection, 5000 cells were cultured with or without Bafilomycin A (5nM; Sigma) and with either no growth factors or cytokines, or with mSCF (50 ng/mL), IL-3 (20 ng/mL), IL-6 (50 ng/mL), Flt3L (25 ng/mL), TPO (25 ng/mL), and GM-CSF (10ng/mL). Cells were cultured for 12 hours and subsequently processed. Caspase activity was detected using the luciferase-based Caspase Glo 3/7 assay system as per manufactures instructions (Promega). Reactive-oxygen species were detected by flow cytometry using the CellROX Deep Red reagent (Life Technologies). For autophagy detection assays, 5000-8000 cells were cultured with or without LY294002 (20uM; Cell Signaling Technologies) and with or without aforementioned growth factors or cytokines.

Autophagy activity was determined by flow cytometry using the CytoID autophagy detection kit as per manufacturers instructions (Enzo Life Sciences).

Table S1, related to *DNA Constructs* in Experimental Procedures. Sequences for FOXO3 shRNA constructs. These sequences were cloned into the MSCV-eGFP retroviral vector to silence FOXO3 expression in bone marrow cells.

FOXO3 shRNA sequence (in miR-155-arms-and-loop-format)	
mmu-FOXO3 shRNA #1	gaaggctgtaTGCTGCCATCATTGAGATTGCTGGTGGTTTGGCCACTGACTGACCACCATGACTGAATGATGGcaggacacaaggcctg
mmu-FOXO3 shRNA #2	gaaggctgtaTGCTGAACACGGTACTGTTGAAGGAGGTTTGGCCACTGACTGACCTCCTTCAAGTACCGTGTTcaggacacaaggcctg
mmu-FOXO3 shRNA #3	gaaggctgtaTGCTGAAGAGAAGGTGGCTGGTCTGTGTTTGGCCACTGACTGACACAGACCACACCTTCTCTTcaggacacaaggcctg

Table S2, related to *Expression profiling and qPCR* in Experimental Procedures.

Primer sequences used for qPCR to quantify expression of various genes. These forward (F) and reverse (R) primers were used to quantify mRNA expression of various genes using RT-qPCR.

Primer sequences

AchE F	CTCCCTGGTATCCCCTGCATA
AchE R	GGATGCCCAGAAAAGCTGAGA
BTG2 F	ATGAGCCACGGGAAGAGAAC
BTG2 R	GCCCTACTGAAAACCTTGAGTC
FOXO1 F	CCCAGGCCGGAGTTTAACC
FOXO1 R	GTTGCTCATAAAGTCGGTGCT
FOXO3 F	CTGGGGGAACCTGTCCTATG
FOXO3 R	TCATTCTGAACGCGCATGAAG
HMGA2 F	GAGCCCTCTCCTAAGAGACCC
HMGA2 R	TTGGCCGTTTTTCTCCAATGG
JARID1A F	CACAGACCCGCTGAGTTTTAT
JARID1A R	CTTCACAGGCAAATGGAGGTT
Lin28b F	AGAATGCAGTCTACCTCCTCAG
Lin28b R	CCTCCCACTTCTCTTGGTGC
MAPK1 F	GGTTGTTCCCAAATGCTGACT
MAPK1 R	CAACTTCAATCCTCTTGTGAGGG
MMP9 F	CTGGACAGCCAGACACTAAAG
MMP9 R	CTCGCGGCAAGTCTTCAGAG
p21 F	CCGCTGGAGGGCAACTTCGT
p21 R	TTTCGGCCCTGAGATGTTCC
p27 F	TCTCAGGCAAACCTCTGAGGAC
p27 R	TTCGGAGCTGTTTACGTCTGG
p300 F	CTCGCACTTGCCCTTACCTTT
p300 R	GGTCGCAGTGGCTGGAGA
p57 F	TGATGAGCTGGGAACCTGAGCC
p57 R	ACGTCGTTCGACGCCTTGTTT
PTBP2 F	GGATCTGACGAGCTACTCTCA
PTBP2 R	TTCTTACTATCGTTACCGTTGGC
RB1 F	TGCATCTTTATCGCAGCAGTT
RB1 R	GTTCACACGTCCGTTCTAATTTG
SOX4 F	GACAGCGACAAGATTCCGTTC
SOX4 R	GTTGCCCCGACTTCACCTTC
SOX5 F	CCCGTGATCCAGAGCACTTAC
SOX5 R	CCGCAATGTGGTTTTTCGCT

Supplemental References

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol* *11*, R106.

Friedman, R.C., Farh, K.K., Burge, C.B., and Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* *19*, 92-105.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* *10*, R25.

Picelli, S., Bjorklund, A.K., Faridani, O.R., Sagasser, S., Winberg, G., and Sandberg, R. (2013). Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat Methods* *10*, 1096-1098.

Roberts, A., and Pachter, L. (2013). Streaming fragment assignment for real-time analysis of sequencing experiments. *Nat Methods* *10*, 71-73.

Satija, R., Farrell, J.A., Gennert, D., Schier, A.F., and Regev, A. (2015). Spatial reconstruction of single-cell gene expression data. *Nature biotechnology*.

Ucar, A., Gupta, S.K., Fiedler, J., Erikci, E., Kardasinski, M., Batkai, S., Dangwal, S., Kumarswamy, R., Bang, C., Holzmann, A., *et al.* (2012). The miRNA-212/132 family regulates both cardiac hypertrophy and cardiomyocyte autophagy. *Nat Commun* *3*, 1078.